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PRINCIPAL INVESTIGATOR: Ari Elson, Ph.D.

CONTRACTING ORGANIZATION: The Weizmann Institute of Science
76100 Rehovot, Israel

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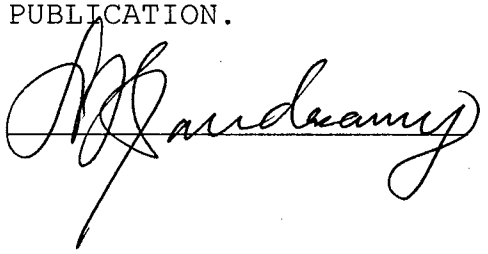

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Ari Elson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Weizmann Institute of Science
76100 Rehovot, Israel
E-MAIL: ari.elson@weizmann.ac.il

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13. ABSTRACT *(Maximum 200 Words)*

In this annual report we describe construction of substrate-trapping mutants of protein tyrosine phosphatase epsilon (PTPε) and the first round of experiments aimed at identification of physiological substrates of PTPε. Mutant PTPε molecules fused to glutathione-S-transferase (GST) were constructed, as were vectors for expressing mutant PTPε molecules in eukaryotic cells. GST fusion proteins could not be isolated in the absence of degradation; eukaryotic expression vectors, however, allowed us to appropriately express mutant PTPε molecules in transfected cells. Use of these mutants enabled us to establish that alpha subunits of voltage-gated potassium (Kv) channels are physiological substrates of PTPε and can be trapped by substrate-trapping mutants of PTPε. Furthermore, work described here provided a much-needed opportunity for calibrating and perfecting technical procedures for trapping substrates with these mutants. This experience will be implemented in future searches for PTPε substrates, searches guided by "educated guesses" of the identities of possible substrates of PTPε, or performed as general screens of mammary tumor cell lines.

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FOREWORD

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5. Introduction:

Our research focuses on providing molecular-level understanding the role of protein tyrosine phosphatase epsilon (PTPε) in the genesis of breast cancer by identifying physiological substrates of the enzyme. We have previously demonstrated a link between PTPε and genesis of mammary tumors in mice *in vivo*; the project supported by the USAMRMC is aimed at taking these findings one step further by defining what is one of the most basic items of information needed to understand the role an enzyme - the identities of its substrates. We aim to achieve this goal through use of novel substrate-trapping methodology, i.e., by use of PTPε mutants which have lost most of their catalytic ability but are able to bind their phosphorylated substrates and remain bound to them during purification. The exact theoretical details of how to construct such mutants have been published in the scientific literature and are briefly summarized below; our aim is to apply this knowledge to PTPε, and to identify substrates of the enzyme in the context of breast tumor cell lines. Specifically, we aim to construct and characterize the necessary mutant PTPε molecules, to identify proteins which bind PTPε substrate-traps or to verify that candidate substrates of PTPε indeed bind substrate-trapping mutants of PTPε, to verify that molecules which bind are indeed substrates of PTPε, and to understand how dephosphorylation by PTPε affects their function. In this first annual report we describe generation of the appropriate mutants and preliminary studies we have performed with them.

6. Body of report:

Brief overview of substrate-trapping technology:

Substrate-trapping mutants of PTPases are PTPase molecules in which specific mutations have been introduced such that the resulting molecules are either almost or entirely devoid of catalytic activity, but retain the ability to bind their phosphorylated substrates and to remain bound to them (Sun et al., 1993; Flint et al., 1997). Binding is typically strong enough to withstand the rigors of immune-precipitation or related procedures, making substrate-trapping mutants "affinity reagents" of sorts for identifying physiological substrates of the phosphatase at hand. Two generic substrate-trapping mutations in PTPases have been described - mutation of the cysteine residue located at the core of the catalytic domain to a serine residue (C-to-S mutation), or mutation of a nearby aspartic acid residue to an alanine (D-to-A)(Flint et al., 1997; Sun et al., 1993). Both residues are strictly conserved in all known PTPases, making this approach systematically applicable to the entire PTPase family. We have undertaken to construct D-to-A mutants of PTPε, either as glutathione-S-transferase (GST) fusion proteins or as proteins which can be exogenously expressed in cells. Both types of reagents are useful to similar extents in such studies, although the technical details of their production and utilization differ somewhat.

Studies planned for the period covered by this report:

During the period covered by this report, which constitutes the first year of the funding period, we undertook to develop substrate-trapping mutants of PTPε and to perform an initial series of experiments with them aimed at identifying molecules trapped by these mutants. The Research Proposal described both GST-(D-to-A)-PTPε fusion proteins as well as D-to-A PTPε cDNAs which can be exogenously expressed in transfected cells, and identified GST fusion proteins as our first tool of choice. We planned to use GST-PTPε proteins in "pull-down" experiments, i.e., addition of GST-PTPε to lysates of cells or tissues, followed by re-isolation of the GST fusion proteins and any molecules which bound them. An alternative approach for the same basic goal was construction of eukaryotic vector plasmids for expression of mutant, substrate-trapping PTPε molecules in cultured cells. Substrate-trapping mutants of PTPε were to be immune-precipitated together with molecules which bound them.

Studies performed during the period covered by the report:

A. Construction of GST-PTPε fusion molecules:

Bacterial expression vectors in which the entire cytoplasmic domain of tm-PTPε, containing both catalytic domains (D1&D2), or just the membrane-proximal (D1) catalytic domain of the enzyme were constructed in the pGEX2TK plasmid (Pharmacia). The required fragments of PTPε were amplified from tm-PTPε cDNA (clone 58, Elson and Leder, 1995a) by PCR, using the e5common oligomer (GGGGATCCAGGTTCCGGAAGCAGAGGA), together with either the AEF3.1 oligomer (TGAACTCTTCCTCCAGCCCG, for the D1 construct) or the Y695/NHE oligomer (GGGCTAGCTCATTTGAAATTAGCATA, for the D1&D2 construct) (See Figure 1). D-to-A mutants, in which the aspartic acid of the D1 domain was mutated to an alanine, were created by site-directed mutagenesis (Kunkel, 1985). The relevant DNA

fragments were cloned into the BamHI/SmaI sites of pGEX2TK; the resulting plasmids were expressed in the BL-21 strain of *E. coli* bacteria.

GST-PTPε fusion proteins were purified from crude bacterial lysates with the use of glutathione-agarose beads (Pharmacia). Expression of GST-PTPε fusion proteins was verified by protein blotting of extracts of bacteria with anti-PTPε antibodies, and by Coomassie blue staining of SDS-polyacrylamide gels through which the lysates or purified proteins had been electrophoresed (Figure 2). In general, protein blot analysis revealed that the GST fusion proteins indeed reacted with anti-PTPε antibodies, indicating that the bacterial expression vectors drove expression of the correct protein. However, Coomassie blue analysis of the purity and of the size of the GST-fusion proteins indicated that full-length GST-PTPε proteins were not being produced in an adequate manner. The protein preparations contained, in addition to full-length GST-PTPε protein, shorter proteins which were likely products of specific cleavage and non-specific degradation (Figure 2). Problems of this nature are often associated with production of GST fusion proteins, especially long ones, as was the case here.

Difficulties of this nature may be overcome by optimizing growth conditions of the bacteria in which the GST fusion proteins are produced. We attempted to grow the GST fusion proteins in BL21 and BL26 bacteria, the *E. coli* strains recommended by the manufacturer (Pharmacia), as well as in other bacteria strains, such as DH5 and XL-1-Blue. We also attempted to limit protein degradation by slowing down the rate of bacterial growth. This was done by growing bacteria in relatively poor media, at temperatures lower than the standard 37 degrees, or by reducing shaking (and aeration) of the bacterial cultures. None of these attempts at optimizing production of GST-PTPε proteins significantly improved the situation. We currently can produce GST-PTPε fusion proteins which contain some full-length product together with significant amounts of degraded material. As our substrate-trapping reagents are key to the success of this project, we felt that we should attempt to produce better reagents for initial substrate-trapping, although we may use our GST-PTPε proteins in the future for studies of a confirmatory nature.

B. Construction of D-to-A mutants of PTPε in eukaryotic expression vectors.

Due to the difficulties encountered in production of GST-(D-to-A)PTPε, we turned to constructing eukaryotic vectors for expressing D-to-A PTPε in eukaryotic cells. The original Research Proposal made provisions for following this course of action should the need arise.

The desired D-to-A mutations were inserted into the cDNAs of the two major forms of PTPε - transmembranal PTPε (tmPTPε, Elson and Leder, 1995a) and cytoplasmic PTPε (Elson and Leder, 1995b). Mutations were inserted using the Kunkel technique for site-directed mutagenesis (Kunkel, 1985); presence of the desired mutations and absence of other sequence changes were verified by sequencing. Both mutant cDNAs (D302A tm-PTPε and D245A cyt-PTPε) were cloned into the pCDNA3 expression vector, and their expression in 293 and COS cells was verified by transient expression and protein blotting with anti-PTPε serum (Elson and Leder, 1995a) (Figure 3).

D-to-A mutants of tyrosine phosphatases are known to be virtually inactive (Flint et al., 1997). In order to determine whether this was the case with our series of D-to-A mutants of PTPε, we compared total tyrosine-phosphatase activity in cells into

which we had transfected wild-type PTPε or D-to-A mutants of PTPε (Figure 4). Briefly, experiments were performed as follows:
* 293 cells were transiently transfected by the calcium-phosphate technique, with the relevant PTPε cDNAs in the pCDNA3 backbone.
* Following lysis in an NP-40-based lysis buffer, protein content of the lysates was measured. Equal amounts of protein were incubated together with paranitrophenyl-phosphate (PNPP), a substrate for phosphatase activity. Dephosphorylation of PNPP yields a product which absorbs light at 405nm; phosphatase activity was then followed as the change in OD405 as a function of time elapsed (up to 120 minutes).
* PNPP can be the substrate also of non tyrosine-specific phosphatases. To correct for this, each experiment was repeated also in the presence of 1mM sodium pervanadate, a powerful, irreversible, and specific inhibitor of tyrosine phosphatases (Huyer et al., 1997; Flint et al., 1997). The rate of PNPP dephosphorylation in the presence of pervanadate was taken to represent activity of non tyrosine-specific phosphatases and was subtracted from the total activity measured without pervanadate.
* Samples containing equal amounts of protein from all cell lysates were examined by protein blotting with anti-PTPε antibodies to verify equal expression levels of the transfected proteins and to ensure that differences in PTPase activity were not due simply to variable expression levels of PTPε in the transfected cells.

As seen in Figure 4, all D-to-A mutants of tm- or of cyt-PTPε were entirely inactive, in agreement with our expectations. Presence of a FLAG tag at the extreme carboxy-terminus of PTPε molecules did not affect catalytic activity. Similar results were later obtained in assaying the ability of wild-type or of mutant PTPε to inhibit activity of voltage-gated potassium channel activity in *Xenopus* oocytes (see below).

C. Use of D-to-A mutants of PTPε:

Identification of voltage-gated potassium channels as physiological substrates of PTPε. In parallel to the research effort described above, we had been characterizing the phenotype of PTPε-deficient, knockout mice we had produced and had come across a defect in myelination of axons in their peripheral nerve system. We had evidence to suggest that this defect was due to aberrant phosphorylation of voltage-gated potassium (Kv) channels in Schwann cells of these mice, and wished to use the substrate-trapping system described above to determine whether Kv channels were in fact substrates of PTPε. Although the studies which led to this point fall outside the scope of the current project, we felt that use of substrate-trapping mutants was nonetheless highly relevant to the goals of the study reported here. The Kv channel study provided us with an excellent opportunity to verify the functionality of the reagents we had constructed and of our operating procedures, using a defined candidate substrate of PTPε, which preliminary evidence indicated was very likely to turn out to be a physiological substrate of PTPε. Furthermore, Kv channels are quite widespread and are expressed in many organs and tissue types which are not conceived as being electrically excitable (Lewis and Cahalan, 1995), raising the possibility that Kv channels may be relevant substrates of PTPε also in the context of breast cancer. In what follows we outline our studies in this direction, focusing in particular on the role of PTPε substrate-trapping mutants.

Previous findings relating to Kv channels in PTPε-deficient mice: In the process of characterizing PTPε-deficient mice we performed an electron microscopy-based study of

the thickness of myelin sheaths surrounding axons in the sciatic nerves of these mice. The original rationale for performing this study was based on knowledge that myelination of peripheral nerves is dependent upon the well-being and functioning of Schwann cells, that Schwann cell proliferation and function is strongly influenced by the functioning of Kv channels expressed in them, and that Kv channels, in turn, can be regulated by Src- and Fyn-mediated tyrosine phosphorylation (Peretz et al., 1999). Several studies documenting functional connections between Src and Fyn, on the one hand, and PTPα, a PTPase extremely closely related to PTPε, on the other hand, have been published (Zheng et al., 1992; Su et al., 1999; Ponniah et al, 1999). This suggested to us that perhaps PTPε could be involved in regulation of Kv channel function in Schwann cells, and that this may be manifested in abnormal myelination of axons.

We first established that primary Schwann cells do in fact express PTPε (Figure 5). We then examined the extent of myelination in PTPε-deficient sciatic nerve axons and found it to be severely reduced compared to wild-type samples (Figure 6). Further studies revealed that alpha subunits of Kv channels in primary Schwann cells derived from PTPε-deficient mice were hyperphosphorylated when compared to age-matched controls (Figure 7). Furthermore, patch-clamp measurements performed on primary Schwann cells revealed that Kv channel activity was markedly elevated in PTPε-deficient cells (Figure 8). Together, these results indicated that lack of cyt-PTPε caused hyperphosphorylation and up-regulation of Kv channels, and most likely accounted for reduced myelination observed *in vivo*.

From these findings we deduced that a normal role of cyt-PTPε in Schwann cells was to lower tyrosine phosphorylation levels and activities of Kv channel alpha-subunits. This conclusion was strengthened by the ability of cyt-PTPε to reduce Src-mediated phosphorylation of Kv2.1 *in vivo* (Figure 9). PTPε could presumably act either directly, by dephosphorylating Kv channel proteins, or indirectly, by dephosphorylating and inactivating kinases (chiefly Src and Fyn), which phosphorylate and activate Kv channels (Peretz et al., 1999). Further studies revealed that activities and phosphorylation levels of Src and of Fyn were similar in Schwann cells of PTPε-deficient and of wild-type mice (not shown), strongly suggesting that Src and Fyn were not major mediators of the effect of cyt-PTPε on Kv channels. This raised the possibility that PTPε directly dephosphorylated Kv channel alpha subunits.

Use of PTPε substrate-trapping mutants: In order to determine whether Kv channel alpha subunits were in fact substrates of PTPε, we made use of the D-to-A mutants of PTPε described above. The purpose of this experiment was to determine whether the major Kv channel alpha-subunit found in Schwann cells, Kv2.1, would specifically bind the D-to-A mutant of PTPε at the latter's active site. For this purpose, we transiently expressed Kv2.1 in 293 cells together with wild-type or the D-to-A mutant of PTPε. PTPε was immune-precipitated using anti-FLAG antibodies, and presence of associated Kv2.1 was examined by protein blotting with anti-Kv2.1 antibodies. As seen in Figure 10, low levels of Kv2.1 reproducibly precipitated with wild-type PTPε. However, significantly more Kv2.1 co-precipitated with the D-to-A mutant of PTPε, indicating that the D-to-A mutation had significantly increased the ability of PTPε to bind Kv2.1. Furthermore, this increased binding was eliminated by the presence of sodium pervanadate in the precipitation reaction. This last finding is critical, as pervanadate oxidizes the cysteine residue located at the catalytic center of PTPases; in the

case of D-to-A mutants, this is known to prevent association between the active site of the mutant molecules and their putative substrates (Flint et al., 1997). Together, these experiments clearly demonstrate that the interaction between Kv2.1 and PTPε is mediated by the active site of the phosphatase, a finding which is clearly consistent with Kv2.1 being a substrate of PTPε.

In line with the above results, expression of wild-type PTPε together with Kv2.1 in *Xenopus* oocytes severely reduced voltage-gated potassium currents caused by Kv2.1 (Figure 11). However, expression of the D-to-A mutant of PTPε in similar experiments had little effect on Kv2.1 activity (Figure 11). This indicates that the D-to-A mutant PTPε molecule is in fact catalytically inactive also as judged in a functional assay which is more specific and physiological than the PNPP dephosphorylation assay described above. Residual reduction in Kv2.1 activity caused by D-to-A PTPε can be readily explained by mutant PTPε binding to some Kv2.1 molecules and thus preventing them from functioning. This effect is expected to be much weaker than enzymatic dephosphorylation of Kv2.1 molecules by active PTPε, as the latter effect is catalytic, while binding is a stoichiometric process in which each PTPε molecule can affect only a single Kv2.1 molecule.

In all, studies performed during this past year have been useful and relevant for future use of D-to-A mutants of PTPε in studies of breast cancer because:

A. We have constructed D-to-A substrate-trapping mutants of PTPε; these mutants are key reagents, without which future work would have been impossible.

B. We have had the opportunity to demonstrate that the D-to-A mutants of PTPε actually function as expected both *in vitro* and *in vivo*, and are appropriate tools for performing the studies we have planned.

C. We have had the opportunity to work out experimental difficulties associated with the techniques of immune-precipitation using substrate-trapping mutants, in a situation where we felt that we had a good candidate substrate at hand. The detailed technical aspects of the study, while not discussed in detail, are critical, as immune-precipitation experiments are typically quite tricky to perform.

D. From the scientific-biological point of view, the results obtained are relevant for the original purpose set forth in the Proposal, as Kv channels are widely expressed and could conceivably be found also in mammary tumor cell lines. We intend to develop this issue further as explained below.

E. These studies have provided us with an invaluable positive control for future studies in which we will attempt to identify previously-unknown substrates of PTPε.

F. This study has reaffirmed that examining whether candidate substrate molecules are in fact substrates of PTPε is a worthy approach and, provided the "educated guesses" made are reasonable, can significantly speed up the rate at which progress is made.

Due to these considerations, we propose to amend the experimental details by which future work will proceed to include:

1. Examination of whether Kv2.1 or any other known Kv channel alpha subunits of a similar nature against which antibodies are available are co-expressed together with PTPε in mammary tumor cell lines. If so, to determine whether these subunits bind D-to-A mutants of PTPε.

2. To examine whether specific molecules are in fact substrates of PTPε. A prominent candidate substrate of PTPε is Neu, as tm-PTPε is specifically expressed, is tyrosine-phosphorylated, and binds Grb2, in mammary tumors initiated by Neu or Ras (Elson and Leder, 1995a; Toledano-Katchalski and Elson, 1999; Gil-Henn et al., in preparation; Figure 12). Another potential substrate of PTPε is c-Src, as Src is dephosphorylated and activated by PTPα, a PTPase extremely closely related to tm-PTPε (Zheng et al., 1992; Su et al., 1999; Ponniah et al., 1999). Furthermore, Src is often activated in mammary tumors initiated by Neu (Muthuswamy et al., 1994; Muthuswamy and Muller, 1995a, 1995b). Initial steps in this direction will be to determine whether Src or Neu bind D-to-A mutants of PTPε.

3. We intend to proceed also with the original plan of research, which includes search for unknown substrates of PTPε in mammary tumor cell lines according to the procedures elaborated in the original Proposal.

7. Key Research Accomplishments:

* Construction of GST fusion proteins of D-to-A substrate-trapping mutants.
* Construction of D-to-A substrate-trapping mutants of tm-PTPε and of cyt-PTPε, in eukaryotic expression vector form.
* Demonstration that these reagents behave as expected (catalytically inactive *in vitro* and *in vivo*; specifically bind Kv channels).
* Demonstration that alpha-subunits of voltage-gated potassium (Kv) channels are substrates of PTPε.

8. Reportable Outcomes:

Manuscripts:

1. Peretz, A., Gil-Henn, H., Sobko, A., Attali, B and Elson, A. - Hypomyelination and increased activity of voltage-gated potassium channels in mice lacking protein tyrosine phosphatase ε. *(in preparation).*

Abstracts and presentations:

1. Concerted activities of protein tyrosine phosphatase epsilon and tyrosine kinases in regulating activity of voltage-gated potassium channels *in vivo*.
   Ari Elson, Bernard Attali, Asher Peretz, Alex Sobko, and Hava Gil.
   FASEB meeting on kinases and protein phosphorylation. Snowmass, Colorado, USA. July 24-29, 1999. (Poster).
2. Concerted activities of protein tyrosine phosphatase epsilon and tyrosine kinases in regulating activity of voltage-gated potassium channels *in vivo*.
   Lecture presented (by Ari Elson) at the Department of Genetics, Harvard Medical School, Boston, USA, July 30, 1999.
3. Protein tyrosine phosphatase epsilon inactivates voltage-gated potassium channels *in vivo*.
   Ari Elson, Bernard Attali, Asher Peretz, Hava Gil, and Alex Sobko·
   EMBO-FEBS Workshop on Protein Phosphatases and Protein Dephosphorylation. September 19-24, 1999. De-Panne, Belgium. (Oral presentation).

Patents:
N/A (not applicable).

Degrees obtained due to support by this grant:
None (yet: grant is supporting work of individuals listed below, although they have not yet obtained their degrees).

Cell lines/repositories:
N/A

Informatics:
N/A

Funding applied for based on this work:
We have applied for a grant from the Israeli Academy of Sciences to pursue further the Kv-PTPε paradigm established here in aspects which do not overlap with breast cancer studies as outlined above (chiefly in lymphocyte and neural function). Funding is sought at a level of $40,000/yr for four years (if approved).

Employment and research opportunities supported by this grant:
Funds support work performed by two PhD students in the lab - Ms. Hila Toledano-Katchalski (who also receives a stipend funded from this grant), and Ms. Hava Gil-Henn. Limited salary support for Mr. Bernard Danovitch, a research technician who assists our lab work, is also provided by this grant.

9. Conclusions:

Studies performed during the period covered by this report have allowed us to construct eukaryotic expression vectors for exogenous expression in cells of substrate-trapping D-to-A mutants of PTPε. We have also demonstrated that these mutant molecules are catalytically inactive, as expected from published literature which describes D-to-A mutants of PTPases. We have made use of these mutants in the closing phases of another research project, in which we were able to demonstrate that alpha subunits of voltage-gated potassium (Kv) channels are physiological substrates of cyt-PTPε. This finding provided a much-needed mechanistic explanation for our previous finding that myelination is impaired in the sciatic nerves of young PTPε-deficient mice, linking this finding with impaired phosphorylation of Kv channels due to lack of cyt-PTPε. Substrate-trapping reagents developed here were important for that study as they enabled us to unequivocally demonstrate physical interaction between the catalytic site of cyt-PTPε and Kv2.1, a prominent Kv channel alpha subunit present in Schwann cells.

Studies outlined here have allowed us to unequivocally demonstrate that our D-to-A substrate trapping mutants of PTPε in fact function as they were expected and provided us with an important opportunity to calibrate our working procedures on what has now been established as a substrate of PTPε. Furthermore, we now have in hand a positive control for future experiments, as well as a potential substrate for PTPε also in the context of mammary tumor cells.

Based on the studies of the past year we recommend to include two additional topics in this study: determination of whether Kv channels are substrates of PTPε also in mammary tumor cells, and determination of whether other specific candidate substrates of PTPε, most prominently Neu and Src, are in fact substrates of the enzyme in mammary tumor cells.

From the scientific point of view, studies performed here have established a clear role for PTPε *in vivo* in regulation of axon myelination in young mice and have established a paradigm which we hope will also extend to mammary tumors. We have established that alpha subunits of Kv channels are physiological substrates of cyt-PTPε in Schwann cells, and that inappropriate dephosphorylation of these channels reduces their ability to function properly. These studies are the first to demonstrate a link between a specific PTPase and regulation of Kv function *in vivo*, and may ultimately link PTPe function to diseases caused by aberrant myelination of axons.

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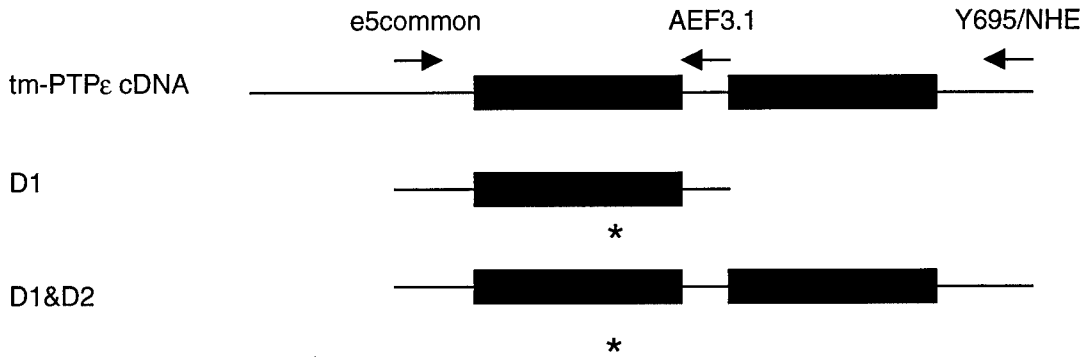

Figure 1: Fragments of tm-PTPε cDNA amplified by PCR for generation of GST-PTPε fusion proteins. Precise sequences of oligonucleotides are given in the text. Black rectangles denote the catalytic domains of PTPε; asterisks denote locations of the aspartic acid mutated to an alanine in D-to-A mutant versions of these constructs.

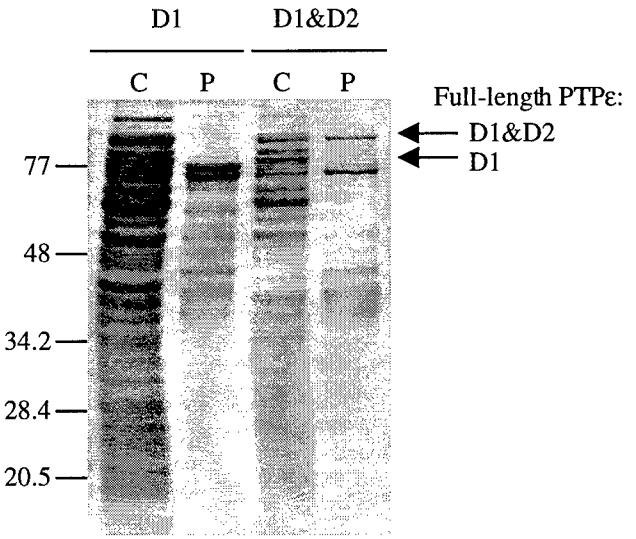

Figure 2: Expression of GST-PTPε fusion proteins. GST fusion proteins of the membrane-proximal catalytic domain of PTPε (D1, construct #308) or of both catalytic domains (D1&D2, construct #304) were constructed as described in the text of the report. Fusion proteins were expressed in BL-21 bacteria and purified using glutathione-agarose beads. Crude bacterial extracts (C) or purified proteins (P) were electrophoresed through a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Arrows denote location of bands of full length GST-PTPε fusion proteins in the lanes containing pure (P) fusion protein in the D1 or D1&D2 lanes. Numbers on the left indicate molecular size markers (in kDa).

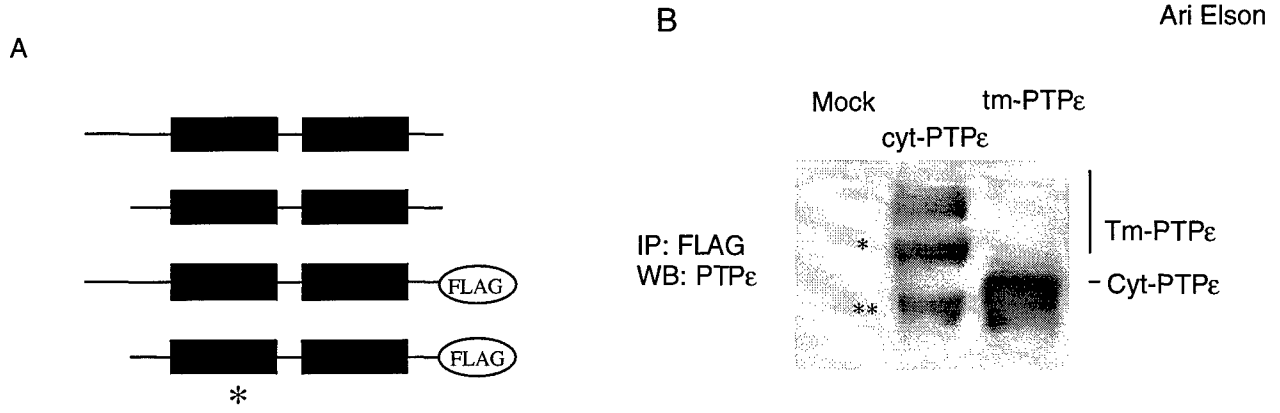

Figure 3: A. Schematic diagram of tm-PTPε and cyt-PTPε cDNAs used in eukaryotic expression studies. Black rectangles denote catalytic domains, ellipses denote FLAG-tag appended to C-termini of some constructs. All cDNAs were cloned into the pCDNA3 expression plasmid. Asterisk marks spot where the D-to-A mutation was inserted. B. Protein blot documenting expression of cDNAs shown in panel A. Proteins were expressed in 293 cells, immune-precipitated using anti-FLAG antibodies,blotted, and probed with an anti-PTPε antibody. Band marked with a single asterisk is unglycosylated tm-PTPε (Elson and Leder, 1995a). Band marked with two asterisks represents a novel, truncated form of PTPε (Gil-Henn et al, in preparation).

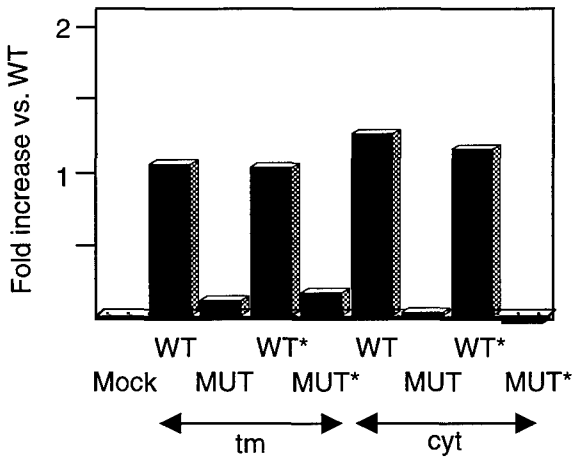

Figure 4: PTPε catalytic activity of wild-type (WT) or D-to-A type mutants (MUT) of tm-PTPε and cyt-PTPε. 293 cells were transfected with the relevant expression vectors, and total PTPase activity, defined as pervanadate-sensitive para-nitrophenylphosphate dephosphorylating activity, was measured. Asterisk denotes FLAG-tagged PTPε. Shown are results of one out of three experiments performed.

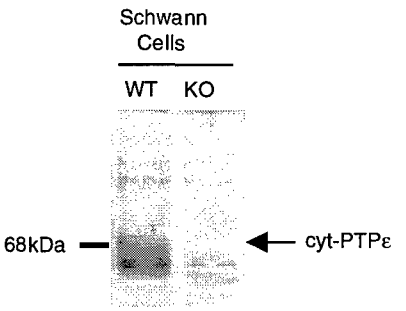

Figure 5: Expression of cyt-PTPε In primary Schwann cells of PTPε-Deficient (KO) or wild-type (WT) mice. Protein extracts of either cell type were electrophoresed and blotted using anti-PTPε antibodies.

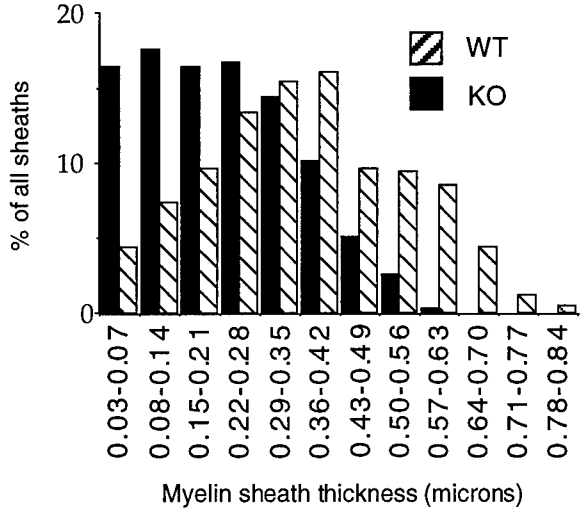

Figure 6: Reduced myelination of axons in sciatic nerves of 5-day old PTPε-deficient (KO, solid) and wild-type (WT, stippled) mice. Cross-sections of sciatic nerve tissue were examined with an electron microscope. Mean sheath thick--nesses were (in microns, +SEM): KO: 0.23±0.05, n=456; WT: 0.37±0.06, n=526. p<0.0001 by the Mann-Whitney test.

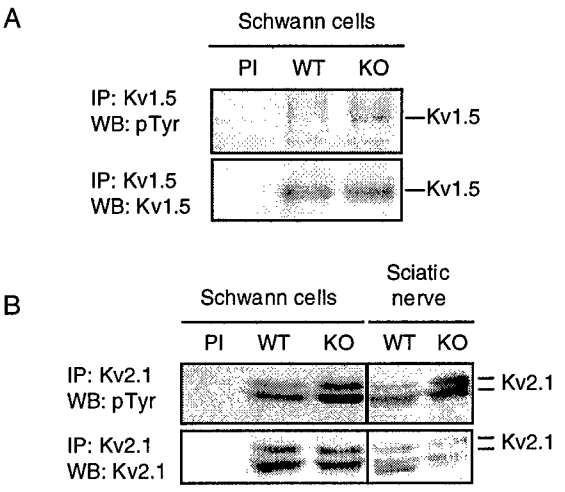

Figure 7: Hyperphosphorylation of Kv channel α-subunits in PTPε-deficient sciatic nerve and primary Schwann cells. Kv2.1 or Kv1.5 were immune-precipitated; blots were probed with anti-phosphotyrosine or anti-Kv antibodies.

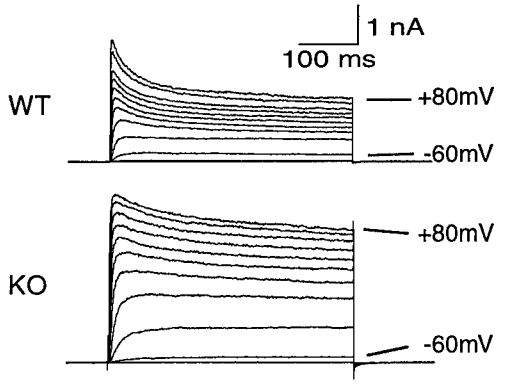

Figure 8: Kv channel activity is upregulated in primary Schwann cells from PTPε-deficient mice. Whole-cell patch-clamp measurements of Kv channel currents induced in wild-type (WT) or PTPε--deficient (KO) cells by successive depolarizations from -60mV to +80mV. Current induced in KO cells is significantly stronger than in WT cells for a given depolarization value. This set of studies was performed in collaboration with Dr. Bernard Attali, of the Department Of Neurobiology of The Weizmann Institute.

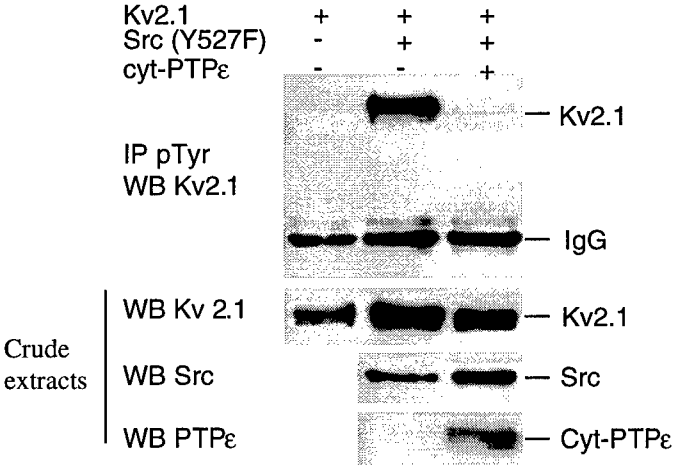

Figure 9: PTPε reduces Src-mediated phosphorylation of Kv2.1. 293 cells were transiently transfected with the indicated expression vectors; tyrosine phosphorylation of Kv2.1 was estimated by immune-precipitating tyrosine-phosphorylated proteins, followed by blotting for Kv2.1 (top). Remaining panels document expression of Kv2.1, Src, or PTPε as indicated.

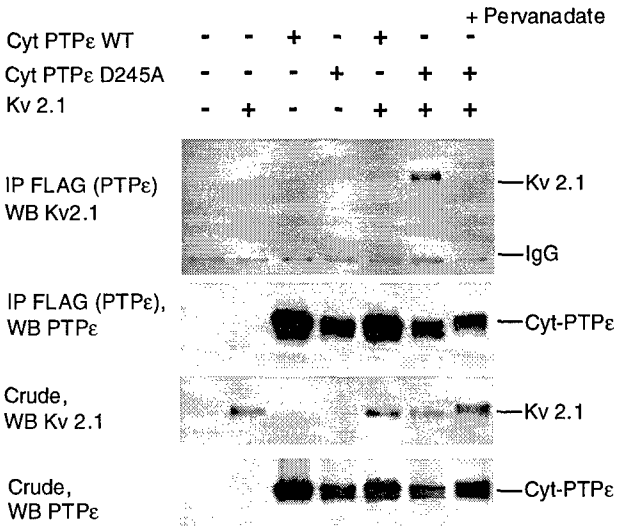

Figure 10: Kv2.1 preferentially binds the D245A substrate-trapping mutant of cyt-PTPε. 293 cells were transfected with WT cyt-PTPε, D245A cyt-PTPε, and Kv2.1 as indicated. Following precipitation of PTPε (via its FLAG tag), precipitates were analyzed for presence of Kv2.1. Note that some Kv2.1 specifically binds wt cyt-PTPε; much more binds D245A cyt-PTPε, and this added binding is removed by pervanadate.

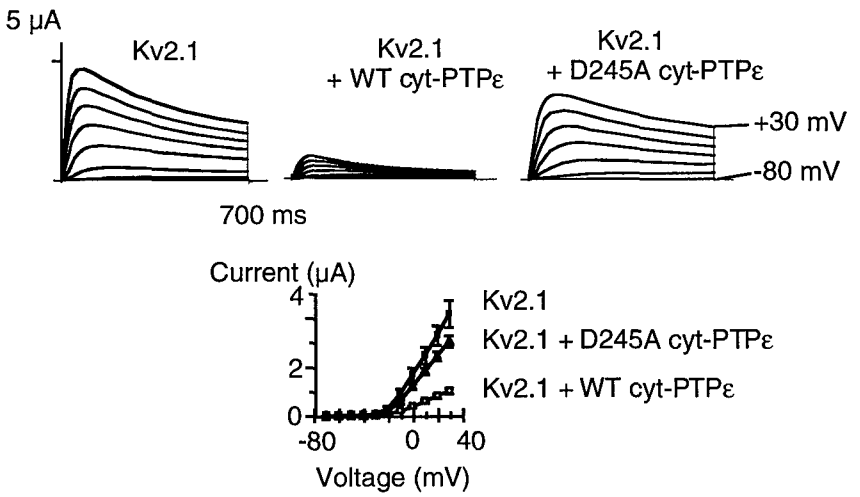

Figure 11: cyt-PTPε reduces Kv2.1-mediated potassium currents in *Xenopus* oocytes. Left: Kv channel currents induced by depolarization in *Xenopus* oocytes expressing Kv2.1. Kv currents are significantly inhibited by co-expression of active cyt-PTPε (middle), but not of a D-to-A mutant of cyt-PTPε (right). Bottom: same data in the form of a graph. Experiment was performed in collaboration with Dr. Bernard Attali.

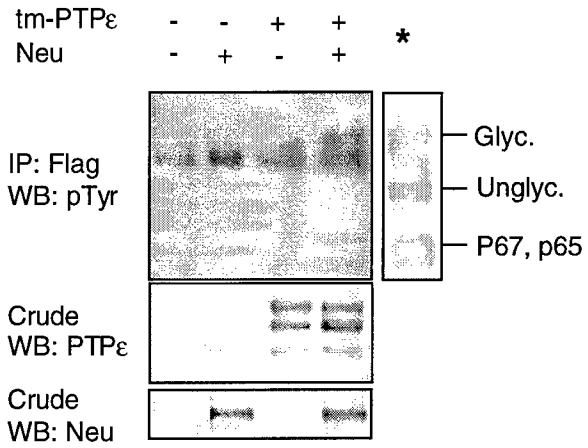

Figure 12: Neu phosphorylates tm-PTPε in Transfected cells. 293 cells were transfected with vectors encoding tm-PTPε or Neu as indicated. Following immunoprecipitation of tm-PTPε - via its FLAG-tag - blot was reacted with anti-phosphotyrosine antibodies. Lane marked with an asterisk shows migration of int-PTPε and of partially-glycosylated tm-PTPε (Elson and Leder, 1995a).

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Nov 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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